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Steelant, Brecht ; Farré, Ricard ; Wawrzyniak, Paulina ; Belmans, Jochen ; Dekimpe, Emily ; Vanheel, Hanne ; Van Gerven, Laura ; Kortekaas Krohn, Inge ; Bullens, Dominique M A ; Ceuppens, Jan L ; Akdis, Cezmi A ; Boeckxstaens, Guy ; Seys, Sven F ; Hellings, Peter W

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# Impaired barrier function in patients with house dust mite-induced allergic rhinitis is accompanied by decreased occludin and zonula occludens-1 expression



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**Background:** Tight junction (TJ) defects have recently been associated with asthma and chronic rhinosinusitis. The expression, function, and regulation of nasal epithelial TJs remain unknown in patients with allergic rhinitis (AR). **Objective:** We investigated the expression, function, and regulation of TJs in the nasal epithelium of patients with house

dust mite (HDM)-induced AR and in an HDM-induced murine model of allergic airway disease.

**Methods:** Air-liquid interface cultures of primary nasal epithelial cells of control subjects and patients with HDM-induced AR were used for measuring transepithelial resistance and passage to fluorescein isothiocyanate-dextran 4 kDa (FD4). *Ex vivo* transtissue resistance and FD4 permeability of nasal mucosal explants were measured. TJ expression was evaluated by using real-time quantitative PCR and immunofluorescence. In addition, the effects of IL-4, IFN- $\gamma$ , and fluticasone propionate (FP) on nasal epithelial cells were investigated *in vitro*. An HDM murine model was used to study the effects of allergic inflammation and FP treatment on transmucosal passage of FD4 *in vivo*.

**Results:** A decreased resistance *in vitro* and *ex vivo* was found in patients with HDM-induced AR, with increased FD4 permeability and reduced occludin and zonula occludens-1 expression. AR symptoms correlated inversely with resistance in patients with HDM-induced AR. *In vitro* IL-4 decreased transepithelial resistance and increased FD4 permeability, whereas IFN- $\gamma$  had no effect. FP prevented IL-4-induced barrier dysfunction *in vitro*. In an HDM murine model FP prevented the allergen-induced increased mucosal permeability. **Conclusion:** We found impaired nasal epithelial barrier function in patients with HDM-induced AR, with lower occludin and zonula occludens-1 expression. IL-4 disrupted epithelial integrity *in vitro*, and FP restored barrier function. Better understanding of nasal barrier regulation might lead to a better understanding and treatment of AR. (J Allergy Clin Immunol 2016;137:1043-53.)

**Key words:** Allergic rhinitis, tight junctions, fluticasone propionate, IL-4, leak pathway, epithelial resistance, epithelial permeability

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A critical role of the airway epithelium is forming a physical barrier protecting the body from inhaled harmful substances. The mucosal barrier is primarily determined by the integrity of intercellular junctions through which epithelial cells are connected to each other (ie, tight junctions [TJs], adherens junctions, and desmosomes), ultimately sealing off the paracellular space.<sup>1</sup> TJs form the most apical intercellular junction between epithelial cells, providing functional polarity between the apical and basolateral domains.<sup>2</sup> TJs consist of different transmembrane proteins, including occludin, tricellulin, the claudin family, and junctional

**Abbreviations used**

ALI:	Air-liquid interface
AR:	Allergic rhinitis
FD4:	Fluorescein isothiocyanate–dextran 4 kDa
FP:	Fluticasone propionate
HDM:	House dust mite
INS:	Intranasal steroid
NEC:	Nasal epithelial cell
RT-qPCR:	Real-time quantitative PCR
TER:	Transepithelial resistance
TJ:	Tight junction
ZO:	Zonula occludens

adhesion molecules.<sup>3</sup> They form intercellular homodimers/heterodimers between neighboring cells, limiting the paracellular passage of macromolecules. Scaffold adaptor proteins, such as cingulin and the zonula occludens (ZO) family, connect the transmembrane proteins to the actin cytoskeleton. TJs are involved in 2 transport pathways: pore and leak pathways.<sup>4</sup> The leak pathway is mainly regulated by occludin and ZO, allowing limited passage of larger molecules, whereas the pore pathway is a size- and charge-selective high-capacity route for ions, as regulated by claudins.

Disturbed TJ function, expression, or both can lead to the entrance of foreign pathogens, irritants, and allergens into the organism.<sup>5</sup> Multiple disorders, such as asthma,<sup>6</sup> inflammatory bowel disease,<sup>7</sup> functional dyspepsia,<sup>8</sup> and atopic dermatitis,<sup>9</sup> have been linked to defective or altered TJ function. Recently, impaired epithelial barrier function was found in patients with chronic rhinosinusitis with or without nasal polyps,<sup>10</sup> suggesting changes in TJ arrangement in the sinonasal mucosa. Nevertheless, the function, expression, and regulation of TJs have not been investigated in patients with allergic rhinitis (AR).

AR is defined as an inflammation of the nasal mucosa resulting from an IgE-mediated allergy to inhaled allergens.<sup>11</sup> AR affects 30% of the Western population and causes symptoms, such as nasal obstruction, rhinorrhea, sneezing, and itchy nose.<sup>12</sup> Despite the available treatment options, such as intranasal steroids (INSs), antihistamines, or leukotriene receptor antagonists and immunotherapy,<sup>13,14</sup> 20% of patients with AR do not respond properly to treatment, representing a therapeutic challenge with a large socioeconomic burden.<sup>15–18</sup> The reason for lack of response to treatment is multifactorial, with barrier dysfunction being one of the reasons. A dysfunctional epithelial barrier might give rise to enhanced uptake of allergens and exogenous particles, leading to more activation of mast cells and nerve fibers.<sup>5,19</sup> Therefore it is of great interest to study epithelial barrier and TJ function in patients with AR.

This study aimed at exploring the function, expression, and regulation of TJs in the nasal epithelium of patients with house dust mite (HDM)–induced AR. Using human air-liquid interface (ALI) cultures of primary nasal epithelial cells (NECs), mucosal explants, and an HDM-induced murine model of allergic airway disease, we demonstrate an impaired barrier in patients with AR and a barrier-correcting effect of fluticasone propionate (FP). FP restored barrier integrity, which might be associated with promotion of expression of occludin and ZO-1.

## METHODS

### Patients

For *in vitro* analyses, NECs were isolated from the inferior turbinates of control subjects ( $n = 16$ ) and steroid-naïve patients with HDM-induced AR

( $n = 9$ ) during aesthetic and/or functional rhinoplasty. Control subjects were nonsmokers, did not report any nasal symptoms suggestive of AR, and did not have a history of AR or rhinosinusitis. All included patients with HDM-induced AR were symptomatic ( $\geq 2$  nasal symptoms of AR) at the time of inclusion, were nonsmoking nonasthmatic subjects, and did not show any clinical sign of infection in the nose at time of surgery. Patient information can be found in Table I.

Nasal biopsy specimens were taken from 10 healthy nonallergic control subjects without any nasal symptoms and from 15 patients with HDM-induced AR to evaluate epithelial barrier integrity at the mucosal explant level. Biopsy specimens were taken 10 minutes after application of a spray with local anesthesia (5% cocaine). Four nasal biopsy specimens were taken with a Fokkens forceps. The biopsy specimens were kept on ice in saline for further analysis. Patients' demographics can be found in Table II.

The study protocol was approved by the local ethics committee of UZ Leuven. The study was registered at [clinicaltrials.gov](http://clinicaltrials.gov) (NCT02461797).

### Evaluation of nasal symptoms

Patients participating in the mucosal explant study were symptomatic ( $\geq 2$  nasal symptoms of AR) and were asked to mark the typical symptoms of AR on a visual analog scale (score, 0–10). The major symptom was selected based on the highest score on a visual analog scale.

### Isolation and ALI cultures of primary NECs and transepithelial resistance measurement

Inferior turbinates were used for isolation of NECs. A highly purified NEC population was obtained, as reported previously.<sup>20</sup> The full methodology is provided in the Methods section in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org).

Freshly isolated NECs were seeded on 0.4- $\mu$ m, 0.33-cm<sup>2</sup> polyester Transwell inserts (Costar, Corning, NY) at a density of  $10^5$  cells per Transwell. Medium was refreshed every other day. Once NECs grew to complete confluence, the apical culture medium was removed to allow further cell differentiation in the ALI. At day 21 in the ALI, epithelial integrity was evaluated by using transepithelial resistance (TER) measurements with an EVOM/Endohm (WPI, Sarasota, Fla). Cultures not building up sufficiently (TER,  $<200 \Omega \times \text{cm}^2$ ) were not included in experiments (14%). TER was measured in triplicate for each subject.

### Paracellular flux measurements

Epithelial permeability as a surrogate marker of layer integrity was measured by using fluorescein isothiocyanate–dextran 4kDa (FD4; Sigma-Aldrich, St Louis, Mo). FD4 (2 mg/mL) was added apically to the ALI cultures at day 21, and the fluorescein isothiocyanate intensity of basolateral fluid was measured with a fluorescence reader (FLUOstar Omega; BMG Labtech, Ortenberg, Germany). FD4 concentration was calculated and is expressed in picomoles (pmols).

### Cytokine stimulation experiments

NEC cultures from control subjects and patients with HDM-induced AR at the ALI were stimulated at day 21 by adding different cytokines to the basolateral compartment: 100 ng/mL IFN- $\gamma$  or 10 ng/mL IL-4 (R&D Systems, Abingdon, United Kingdom). One hour before cytokine stimulation, 0.1  $\mu$ mol/L FP (Sigma-Aldrich) or saline was added. TER and FD4 permeability was measured at 0, 24, 48, and 72 hours after stimulation. The optimal concentration for the different stimulation experiments was based on dose-response experiments in NEC cultures at the ALI.

### Ussing chamber experiments for evaluation of mucosal explant integrity

Nasal biopsy specimens were mounted in Ussing chambers (Mussler Scientific Instruments, Aachen, Germany) with an opening of 0.017 cm<sup>2</sup> to evaluate mucosal integrity *ex vivo*, as described previously.<sup>8</sup> The methodology can be found in the Methods section in this article's Online Repository.

**TABLE I.** Patients' demographic information for *in vitro* cell work

	Control subjects	Patients with HDM-induced AR	P value
No.	16	9	NA
Age (y)	37 ± 18	31 ± 13	.43
Male sex	56	67	.69
Smoking	0	0	NA
INS use	0	0	NA
Asthma	0	0	NA
HDM allergy	0	100	NA

NA, Not applicable.

**TABLE II.** Patients' demographic information for *ex vivo* study

	Control subjects	Patients with HDM-induced AR	P value
No.	10	15	NA
Age (y)	29 (25-33)	24 (21-30)	.14
Male sex	30	29	NA
Smoking (no.)	0	0	NA
INS use (no.)	0	6	NA
Mometasone furoate	0	3	NA
Fluticasone furoate	0	3	NA
Asthma (no.)	0	0	NA
HDM allergy (no.)	0	15	NA

NA, Not applicable.

## HDM-induced murine model of allergic airway disease

Male C57Bl/6 mice (6-8 weeks) were obtained from Harlan (Horst, The Netherlands) and kept under conventional conditions. Experimental procedures were approved by the Ethical Committee for Animal Research at KU Leuven. Mice were endonasally sensitized with 50  $\mu$ L of HDM extract (1  $\mu$ g/mL; Greer Laboratories, Lenoir, NC) or 50  $\mu$ L of saline at day 0. From days 7 to 11, mice were endonasally challenged with 50  $\mu$ L of HDM extract (10  $\mu$ g/mL) or 50  $\mu$ L of saline. One hour before each challenge, mice were administered endonasally FP (0.3 mg/kg body weight) or sham (saline/10% dimethyl sulfoxide). One, 24, or 72 hours after the last HDM challenge, 20  $\mu$ L of FD4 (50 mg/mL) was applied endonasally, allowing evaluation of nasal mucosal permeability. One hour after FD4 application, mice were killed with intraperitoneal injection of Nembutal (Ceva, Brussels, Belgium). Serum and the nasal mucosa were collected. Bronchoalveolar lavage was performed, as previously described.<sup>21</sup> FD4 levels were determined in the serum by using a fluorescence reader (FLUOstar Omega).

## Real-time quantitative PCR for TJ genes

The methods for mRNA isolation and real-time quantitative PCR (RT-qPCR) have been reported previously.<sup>22,23</sup> We chose to evaluate occludin, ZO-1, claudin-1, and claudin-4 expression based on a literature search.<sup>10,24</sup> Details and primer sequences can be found in the [Methods](#) section and [Table E1](#) in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org).

## Immunofluorescence staining of TJs

See the [Methods](#) section in this article's Online Repository for a detailed description of immunofluorescence staining of TJs in ALI cultures and nasal biopsy specimens. Immunofluorescence stainings were performed on at least 8 paraffin slices (5  $\mu$ m) of 8 healthy control subjects and 9 patients with HDM-induced AR. Analysis of TJ immunofluorescence intensity was performed in a blinded manner.

## Statistical analysis

Data were analyzed with GraphPad Prism 5 software (GraphPad Software, La Jolla, Calif). Differences between groups were analyzed by using the 2-tailed unpaired *t* test or Mann-Whitney *U* test. Data are presented as means  $\pm$  SDs or medians (interquartile ranges). One-way ANOVA or the Kruskal-Wallis test with *post hoc* analysis was used to compare multiple groups. Categorical values were compared by using the Fisher exact test. Pearson *r* or Spearman  $\rho$  measurements were used to determine correlations. Values were considered significantly different at a *P* value of less than .05.

## RESULTS

### *Ex vivo* disturbed barrier function in nasal biopsy specimens of patients with HDM-induced AR

We first evaluated epithelial barrier function at the mucosal level in symptomatic patients with HDM-induced AR without treatment (*n* = 9) and control subjects (*n* = 10). For this purpose, nasal biopsy specimens were taken, and mucosal integrity was evaluated in Ussing chambers. Nasal biopsy specimens of patients with HDM-induced AR showed significantly lower transtissue resistance compared with those of control subjects (*P* < .05; [Fig 1, A](#)), with a higher transmucosal FD4 passage (*P* < .05; [Fig 1, B](#)). Transtissue resistance and FD4 passage correlated inversely, both in control subjects (*P* < .01) and patients with HDM-induced AR (*P* < .001; [Fig 1, C](#)).

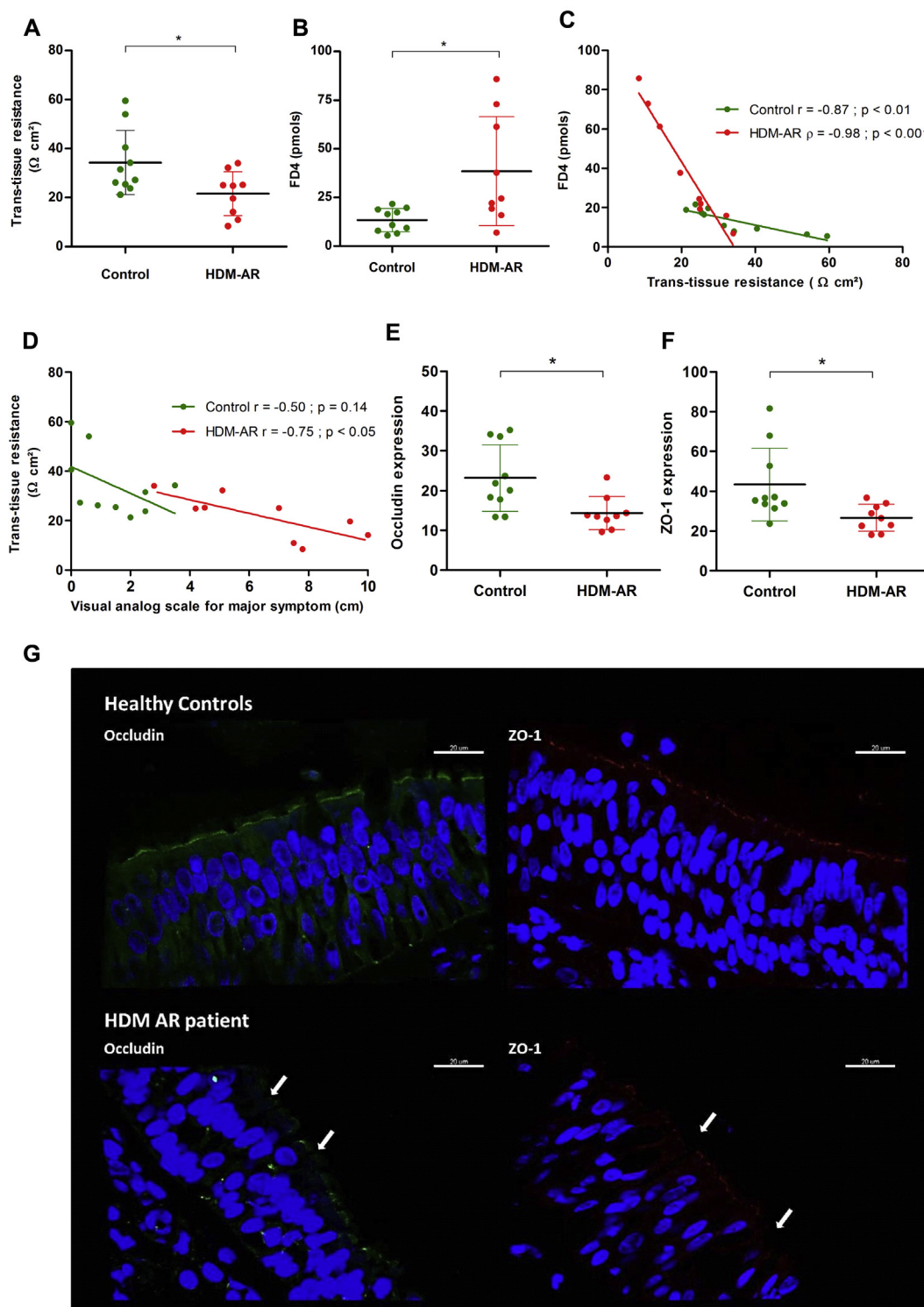
In patients with HDM-induced AR, the visual analog scale for major symptoms correlated inversely with transtissue resistance (*P* < .05; [Fig 1, D](#)), which was not found in control subjects (*P* = .14; [Fig 1, D](#)).

In view of the *ex vivo* evidence for a disrupted epithelial barrier in patients with HDM-induced AR, we investigated the expression of the TJ network. Although more than 60 genes are associated with junctional complexes, we focused on the expression of claudin-1, claudin-4, occludin, and ZO-1. RT-qPCR revealed significantly lower ZO-1 (*P* < .05) and occludin mRNA (*P* < .05) expression in patients with HDM-induced AR compared with that in control subjects ([Fig 1, E and F](#), respectively). No difference in mRNA expression was found for claudin-1 (*P* = .84) and claudin-4 (*P* = .95) between patients with HDM-induced AR and control subjects. Immunofluorescence staining showed a relatively weak arrangement of occludin and ZO-1 in biopsy specimens of patients with HDM-induced AR compared with control subjects ([Fig 1, G](#)). Quantification of mean occludin fluorescence intensity showed a trend toward lower intensity of occludin in patients with HDM-induced AR compared with control subjects without reaching significance ( $6.4 \times 10^8$  vs  $1.1 \times 10^9$ , *P* = .18).

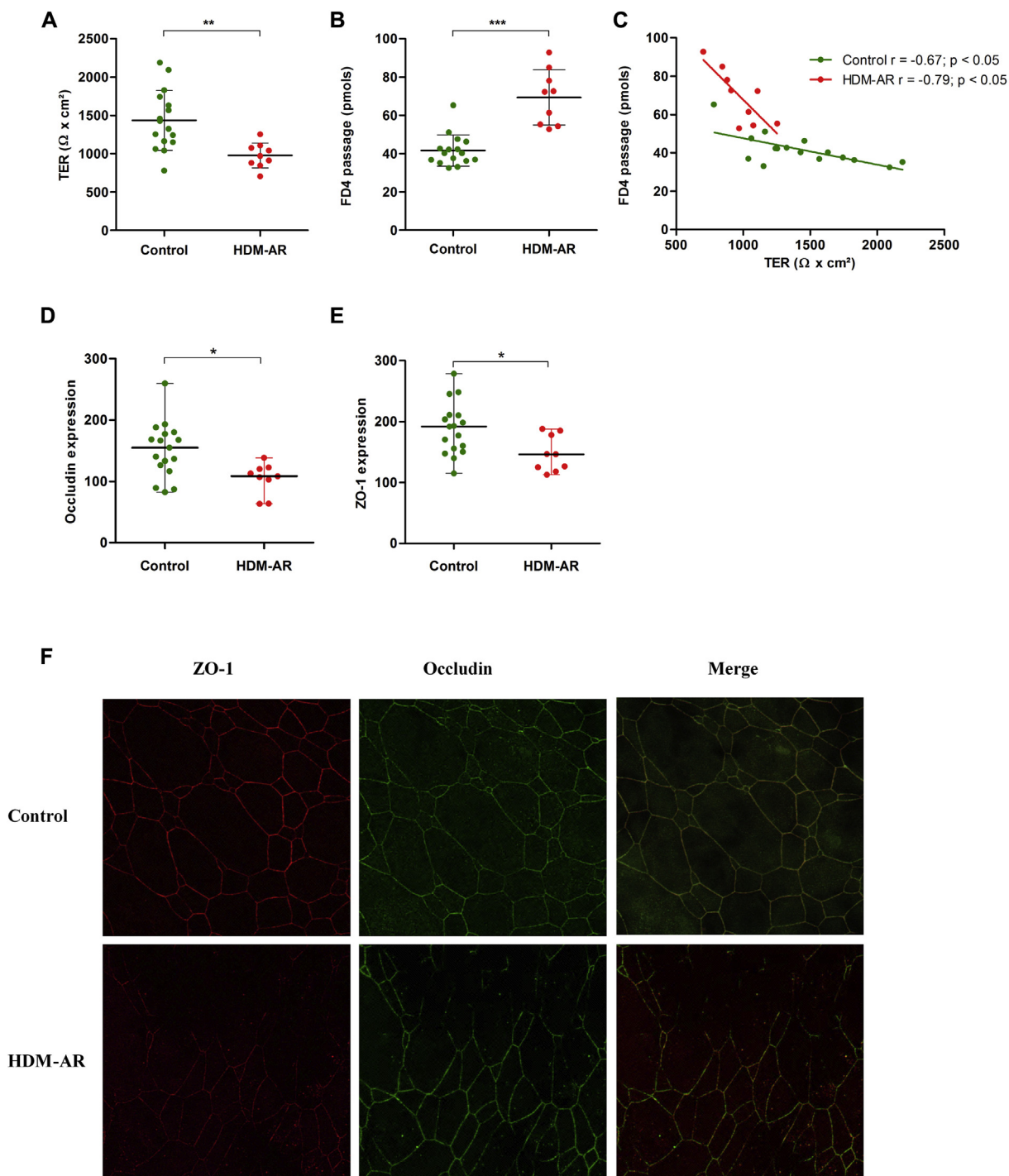
### *In vitro* impaired nasal epithelial barrier function in patients with HDM-induced AR

Next, we studied nasal epithelial integrity *in vitro* in patients with HDM-induced AR and control subjects. Therefore NECs were cultured at the ALI. After 21 days, TER of NEC cultures from patients with HDM-induced AR was significantly lower compared with that of control subjects (*P* < .01; [Fig 2, A](#)). Paracellular flux of FD4 across the NEC monolayer showed a significantly increased passage of FD4 in ALI cultures of patients with HDM-induced AR (*P* < .001; [Fig 2, B](#)). Moreover, TER negatively correlated with FD4 passage, both in NEC cultures

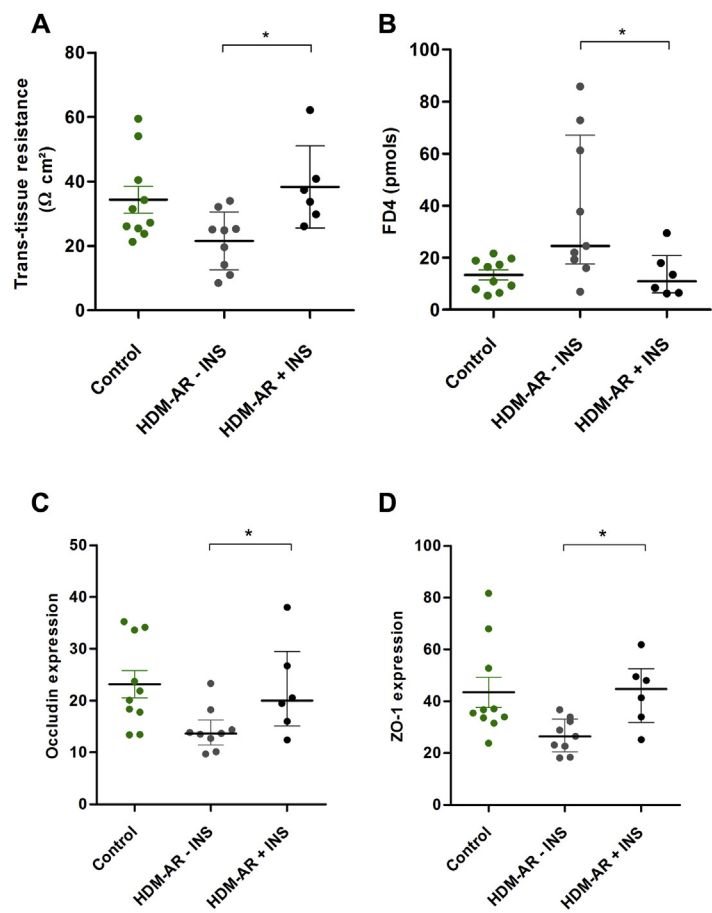




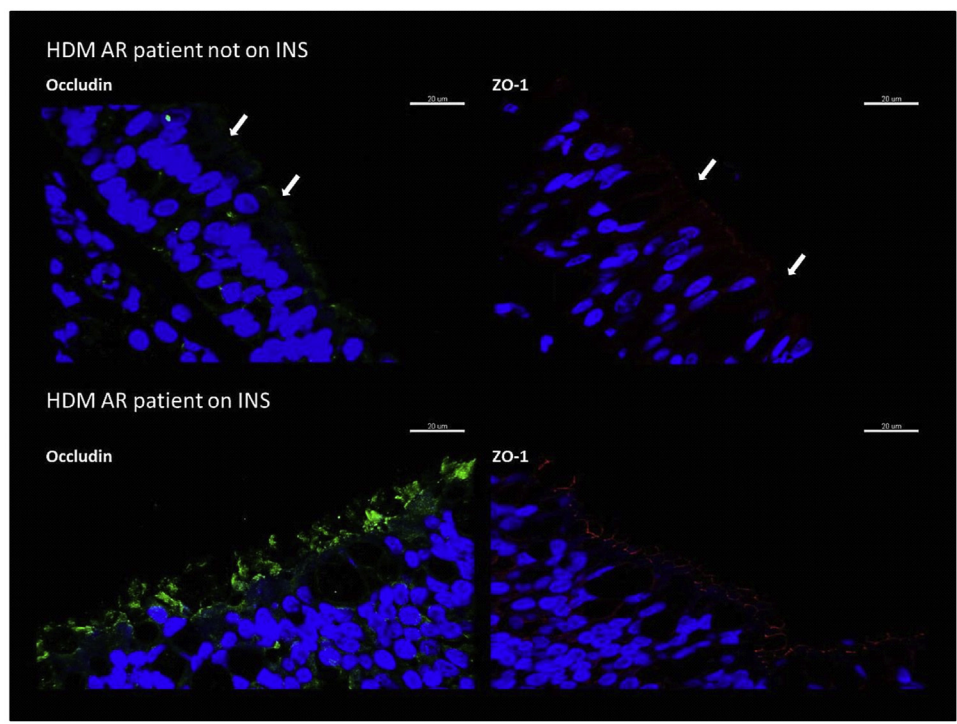
**FIG 1.** Impaired mucosal barrier function *ex vivo* in patients with HDM-induced AR. Nasal biopsy specimens were isolated from control subjects and patients with HDM-induced AR for evaluation of mucosal integrity in Ussing chambers. **A**, Transtissue resistance measured with Ussing chambers. **B**, FD4 epithelial permeability. **C**, Transtissue resistance negatively correlated with FD4 passage in control subjects and patients with HDM-induced AR. **D-E**, Lower mRNA expression of occludin and ZO-1 in patients with HDM-induced AR compared with that in control subjects. Relative mRNA expression versus the housekeeping genes encoding  $\beta$ -actin and  $\beta_2$ -microglobulin is shown. **F**, Transtissue resistance negatively correlated with visual analog scale scores for major symptoms in patients with HDM-induced AR. **G**, Representative immunofluorescence staining for occludin and ZO-1 in 8 control subjects and 9 patients with HDM-induced AR. \* $P < .05$ .



**FIG 2.** Impaired NEC function *in vitro* in patients with HDM-induced AR. NECs were isolated from the inferior turbinate and cultured *in vitro*. **A**, TER in ALI cultures after 21 days in patients with HDM-induced AR and control subjects. **B**, FD4 passage in patients with HDM-induced AR and control subjects. **C**, TER negatively correlated with FD4 passage in control subjects and patients with HDM-induced AR. **D-E**, Lower mRNA expression of occludin and ZO-1 in patients with HDM-induced AR compared with control subjects. Relative mRNA expression versus the housekeeping genes encoding  $\beta$ -actin and  $\beta_2$ -microglobulin is shown. **F**, Representative immunofluorescence staining showed less clear arrangement for occludin and ZO-1 in patients with HDM-induced AR compared with that in control subjects. \* $P < .05$ , \*\* $P < .01$ , and \*\*\* $P < .001$ .



**E**



of control subjects ( $P < .05$ ) and those of patients with HDM-induced AR ( $P < .01$ ; Fig 2, C).

Significantly lower mRNA expression was found in patients with HDM-induced AR for ZO-1 ( $P < .05$ ; Fig 2, D) and occludin ( $P < .05$ ; Fig 2, E) compared with that in control subjects. No difference in mRNA expression was found for claudin-1 ( $P = .74$ ) and claudin-4 ( $P = .33$ ) between patients with HDM-induced AR and control subjects.

Immunofluorescence staining of the TJ proteins occludin and ZO-1 revealed an intact TJ layer in NEC cultures at the ALI of control subjects, with strong expression of occludin and ZO-1 (Fig 2, F). However, the epithelial layer was disrupted in NEC cultures at the ALI of patients with HDM-induced AR, with less structured colocalization of TJs and lower expression of occludin and ZO-1.

Taken together, the *ex vivo* and *in vitro* data showed an impaired epithelial barrier function in patients with HDM-induced AR, which is linked to reduced ZO-1 and occludin expression.

### INSs restored barrier function *ex vivo*

INSs are first-line therapy in patients with AR. We compared nasal mucosal explant function in patients with HDM-induced AR using INSs ( $n = 6$ ) and patients not using INSs ( $n = 9$ ). Nasal biopsy specimens of patients with HDM-induced AR using INSs showed a significantly higher transtissue resistance ( $P < .05$ ; Fig 3, A) and a significantly lower FD4 passage ( $P < .05$ ; Fig 3, B) compared with values in patients with HDM-induced AR not using INSs. Analysis of mRNA expression revealed a higher occludin and ZO-1 expression in patients with HDM-induced AR using INSs (Fig 3, C and D, respectively). Immunofluorescence staining for ZO-1 and occludin showed a more intact barrier in patients taking INSs compared with that in patients with HDM-induced AR not using INSs (Fig 3, E).

### FP prevented IL-4-induced barrier disruption

Because we found *ex vivo* evidence for restored barrier function in patients with HDM-induced AR using INSs, we investigated how INSs, more specifically FP, and cytokines affect barrier function. NEC cultures at the ALI of control subjects ( $n = 5$ ) and patients with HDM-induced AR ( $n = 4$ ) were exposed in duplicate to IL-4 (10 ng/mL) and IFN- $\gamma$  (100 ng/mL) at the basolateral side. One hour before cytokine stimulation, FP (0.1  $\mu$ mol/L) or saline was added to the basolateral medium. Incubation with IL-4 significantly reduced TER in cultures from control subjects (Fig 4, A). When IL-4-supplemented cultures were pretreated with FP, no decrease in TER was found (Fig 4, A). FP alone increased TER after 72 hours ( $P < .05$ ; Fig 4, A). FD4 permeability was significantly increased after 72 hours of incubation with IL-4, whereas pretreatment of the IL-4 cultures with FP significantly reduced FD4 permeability.

No significant decrease in FD4 permeability was found after FP stimulation (Fig 4, B). Similar responses for IL-4, FP, and the combination of IL-4 and FP were obtained in NEC cultures at the ALI in patients with HDM-induced AR ( $n = 4$ , see Fig E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Treatment with IFN- $\gamma$  did not influence the epithelial integrity or permeability in NEC cultures at the ALI in control subjects and patients with HDM-induced AR (Fig 4, C and D, and see Fig E1).

FP incubation for 72 hours upregulated mRNA expression of occludin and ZO-1 (both  $P < .05$ , see Fig E2 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)) compared with medium in NEC cultures at the ALI in control subjects ( $n = 5$ ). FP did not have an effect on mRNA expression of claudin-1 and claudin-4.

Next, a glucocorticoid receptor antagonist, RU-486, was used to antagonize the effect of FP on barrier function. RU-486 alone did not affect barrier integrity (Fig 5) and permeability ( $45 \pm 20$  vs  $35 \pm 12$  pmol,  $P = .38$ ) *in vitro* in NEC cultures at the ALI in control subjects. When RU-486 was added to NEC cultures at the ALI 1 hour before FP, no increase in TER was measured (Fig 5).

### Increased mucosal permeability after challenge with HDM extract in a murine model of allergic airway disease

In a last series of experiments, we used an HDM-induced murine model of allergic airway disease<sup>25</sup> to study mucosal permeability after HDM sensitization and challenge and evaluated the effect of FP treatment on mucosal permeability (Fig 6, A). A significantly higher FD4 passage was found in sham/HDM mice 1 and 24 hours after the last HDM challenge (Fig 6, B) compared with sham/saline mice. FP reduced FD4 passage to the blood in HDM-challenged mice 1 and 24 h after the last challenge. FP alone (without HDM challenge) did not have a significant effect on FD4 passage. No differences between the 4 groups with regard to FD4 passage were found 72 hours after the last HDM challenge (Fig 6, B). To conclude, FP prevented HDM-induced enhancement of FD4 passage in a murine model of HDM-induced allergic airway inflammation.

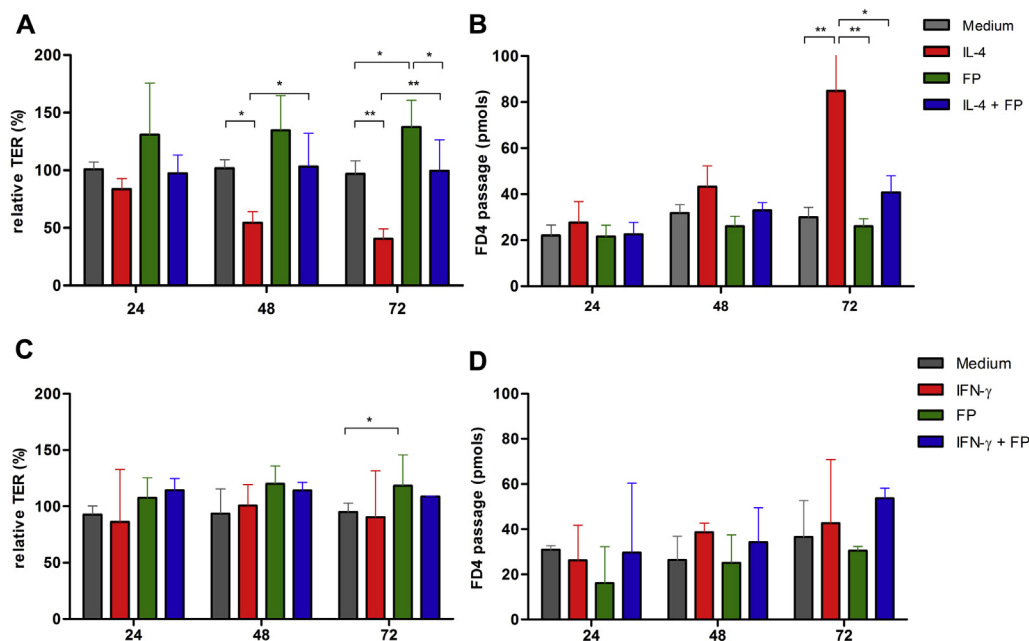
### DISCUSSION

Our study demonstrates impaired epithelial barrier function in patients with HDM-induced AR. A lower epithelial integrity together with an increased permeability and altered occludin and ZO-1 expression was found in patients with HDM-induced AR. First-line treatment with INSs restored mucosal integrity. FP restored IL-4-induced impairment of epithelial barrier function and resulted in upregulation of occludin and ZO-1 mRNA expression.

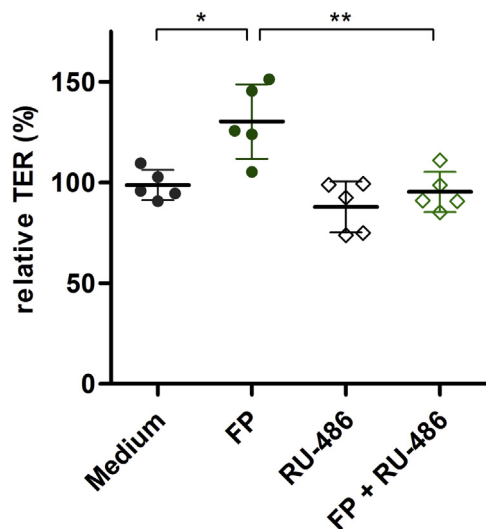
AR is a prevalent inflammatory disorder of the nasal mucosa, with the pathophysiologic mechanisms responsible for the

**FIG 3.** INSs restored barrier function *ex vivo*. Nasal biopsy specimens were isolated from patients with HDM-induced AR using INSs ( $n = 6$ ) and not using INSs ( $n = 9$ ) for evaluation of mucosal integrity in Ussing chambers. **A**, Transtissue resistance measured with Ussing chambers. **B**, FD4 epithelial permeability. **C-D**, mRNA expression of occludin and ZO-1 was increased in patients with HDM-induced AR using INSs. Relative mRNA expression versus the housekeeping genes encoding  $\beta$ -actin and  $\beta_2$ -microglobulin is shown. **E**, Representative immunofluorescence staining showed a restored organization for occludin and ZO-1 in 3 patients with HDM-induced AR using INSs compared with 6 patients with HDM-induced AR not using INSs. The data obtained for control subjects and patients with HDM-induced AR using INSs are duplicated from Fig 1. \* $P < .05$ .





**FIG 4.** Regulation of epithelial barrier function *in vitro* by IL-4, IFN- $\gamma$ , and FP in healthy control subjects. NECs were isolated from the inferior turbinates of control donors ( $n = 5$ ) and cultured *in vitro* to evaluate the effect of proinflammatory cytokines and FP on NEC integrity. **A**, Effect of IL-4, FP, and IL-4 plus FP on epithelial integrity, as measured with TER in duplicate. **B**, Effect of IL-4, FP, and IL-4 plus FP on FD4 epithelial permeability. **C**, IFN- $\gamma$  did not influence TER measurements in control subjects. **D**, No effect of IFN- $\gamma$  on FD4 epithelial permeability in control subjects. \* $P < .05$  and \*\* $P < .01$ .

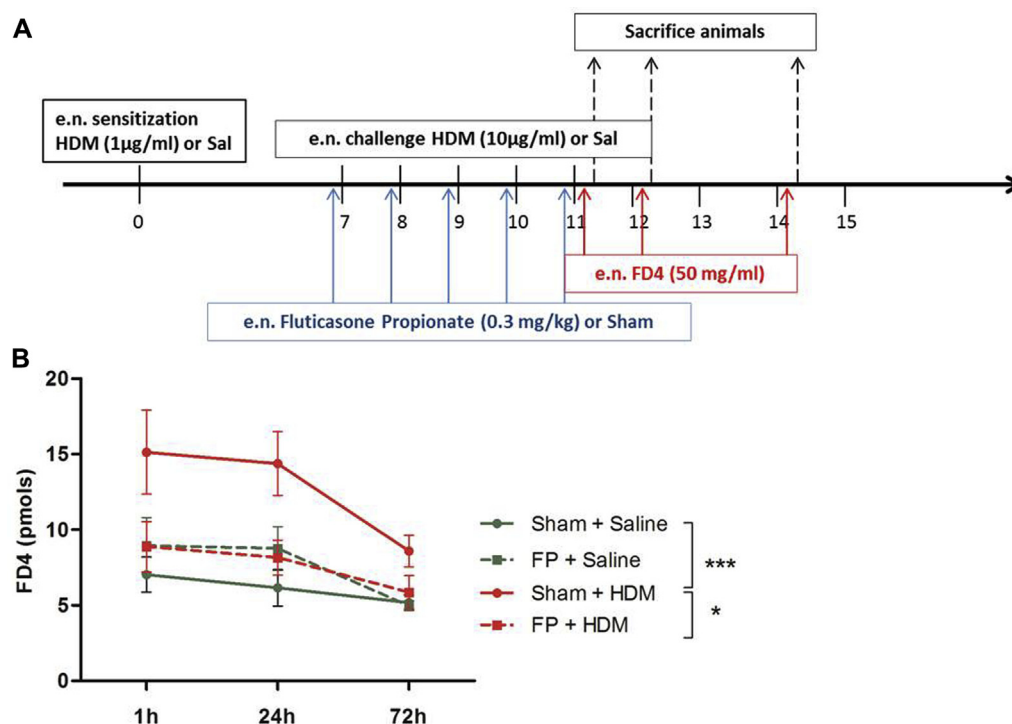


**FIG 5.** RU-486 antagonized the effect of FP on epithelial integrity. NECs were isolated from the inferior turbinates of control subjects ( $n = 5$ ) and cultured *in vitro* to study the effect of FP and its antagonist, RU-486. Relative TER changes in NEC cultures after stimulation. \* $P < .05$  and \*\* $P < .01$ .

severity of disease being only partly understood. In this regard the role of the nasal epithelium in protecting subepithelial cells from exposure to allergens remains unknown. Despite different treatment options, 20% of patients with AR do not respond to current treatment and remain symptomatic.<sup>15</sup> In these patients we are considering barrier dysfunction an important mechanism in understanding their uncontrolled allergic inflammation. In view

of this, lower mRNA expression of occludin and ZO-1 was found in patients with HDM-induced AR at both the cellular and tissue levels. Therefore we conclude that the epithelial leak pathway is involved in the pathology of AR. Less organized arrangement of occludin and ZO-1 was found in patients with AR, suggesting that defects in the leak pathway might facilitate the passage of allergens and environmental proinflammatory agents through the epithelial barrier. Interestingly, the strength of the leak pathway is also reflected in the correlations between epithelial integrity and FD4 permeability (Figs 1, C, and 2, C). Patients with HDM-induced AR had a higher angle of the slope of correlation, illustrating that an increased epithelial permeability, which is found in patients with HDM-induced AR, might give rise to activation of mast cells and afferent nerve fibers with induction of inflammation and symptoms. Indeed, visual analog scale scores for major symptoms correlated with transtissue resistance, pointing to an important mechanism in the pathology of AR.

Our data showing reduced expression of occludin and ZO-1 are in line with observations in patients with chronic rhinosinusitis with nasal polyps,<sup>10</sup> as well as in asthmatic patients,<sup>6</sup> patients with atopic eczema,<sup>9</sup> and patients with inflammatory bowel disease,<sup>7</sup> in whom impaired epithelial TJ function is considered part of the pathophysiology. In contrast to the leak pathway, the pore pathway regulated by claudins does not seem to be of key importance because no changes in claudin-1 and claudin-4 expression were observed in our study. Nevertheless, we cannot exclude the possibility of other claudins being involved because there are now more than 20 claudins identified<sup>26</sup> without precise insight into their contribution to the nasal mucosal barrier. Indeed, we have checked the expression of claudin-2, the major



**FIG 6.** HDM-induced murine model of allergic airway disease to study barrier function *in vivo*. **A**, Schematic representation of the murine model. *e.n.*, Endonasal. **B**, FD4 passage in the serum at different time points. Data are presented as means  $\pm$  SEMs. \* $P < .05$  and \*\*\* $P < .001$ .

pore-forming claudin in the intestine, in patients with HDM-induced AR. There was no upregulation of claudin-2 compared with levels seen in control subjects (data not shown).

A dysfunctional nasal epithelial barrier in patients with AR has been hypothesized to result in excessive allergen exposure and stimulation of inflammatory cells<sup>27,28</sup> on the one hand and clearance of inflammatory mediators from the tissue to the lumen on the other hand. Therefore restoring the epithelial barrier function could limit the entrance of allergens. In the present study we found that patients using INSs on a daily basis had a more intact barrier than non-INS-treated patients, with upregulation of the TJ proteins occludin and ZO-1 in the INS group. These data are fully in line with those of Sekiyama et al,<sup>29</sup> who showed that budesonide, dexamethasone, and FP enhanced barrier function of Calu-3 and 16HBE epithelial cells *in vitro* in a dose- and time-dependent manner. Importantly, our data were obtained by using primary NECs and are therefore more relevant than data obtained by using epithelial cell lines, which have different morphology, biochemical characteristics, and cellular responses than freshly isolated NECs.<sup>30</sup> Alternatively, activation of Toll-like receptor 9 on airway epithelial cells by CpG-DNA can also restore epithelial barrier integrity by affecting TJ expression, highlighting the importance of restoration of epithelial barrier function.<sup>31</sup>

To analyze the effect of inflammation on epithelial barrier function, we used an *in vitro* NEC culture system from control subjects and patients with HDM-induced AR to explore the effects of proinflammatory cytokines: the  $T_H2$  cytokine IL-4 and the  $T_H1$  cytokine IFN- $\gamma$ . IL-4 decreased TER of freshly isolated NECs with enhanced FD4 paracellular passage, as described earlier by others,<sup>10,32</sup> although the effect of IL-4 was

less pronounced in cultures from patients with HDM-induced AR. The limited effect of IL-4 in patients with HDM-induced AR is due to the already leaky epithelium, and therefore IL-4 cannot induce much more damage. The exact mechanism of IL-4 on barrier (dys)function is thus far not fully known. It is suggested that IL-4 might upregulate the expression of RhoA, a small GTPase, which is a molecular switch for TJ homeostasis. Upregulation of RhoA leads to disassembly of TJs and therefore opening of the leak pathway.<sup>33</sup> In contrast to IL-4, IFN- $\gamma$  did not interfere with TER or FD4 passage in our *in vitro* NEC culture system of control subjects and patients with HDM-induced AR. Intriguingly, IFN- $\gamma$  has previously been reported to increase epithelial permeability in primary bronchial epithelial cells<sup>10</sup> and in T84 colonic epithelial cells,<sup>34</sup> which was not confirmed in our culture system by using freshly isolated NECs. This discrepancy might be explained by the fact that airway epithelial cells are primarily  $T_H2$  sensitive.<sup>35</sup> Indeed, Ahdieh et al<sup>36</sup> have shown that IFN- $\gamma$  enhanced lung epithelial wound healing, illustrating the bivalent effect of IFN- $\gamma$  depending on the cells used.

The *in vitro* NEC cultures were also used to study the effect of one of the most commonly used INSs: FP. In NEC cultures FP restored the IL-4-induced changes in nasal epithelial integrity, which has not been reported before and allows a broader interpretation of the action of INSs. FP treatment alone increased epithelial integrity through upregulation of occludin and ZO-1. Boivin et al<sup>37</sup> have previously shown similar effects in Caco-2 intestinal epithelial cells stimulated with TNF- $\alpha$ .<sup>37</sup> TNF- $\alpha$  has previously been associated with increased intestinal epithelial permeability, as measured with Ussing chambers, in patients with colitis.<sup>38</sup> When Caco-2 epithelial cells were treated with

glucocorticoids, the TNF- $\alpha$ -induced increase in TJ permeability was reversed.<sup>37</sup> On the other hand, IL-1 $\beta$  and IFN- $\gamma$ /TNF- $\alpha$  in combination with dexamethasone did not result in decreased permeability.<sup>39</sup> This discrepancy might be explained by differences in culture procedures and cytokine and/or glucocorticoid concentrations.

Lastly, we evaluated *in vivo* whether HDM-sensitized mice showed increased nasal permeability after HDM challenge. Significantly higher serum FD4 levels were found in HDM-sensitized animals 1 and 24 hours after the last allergen challenge, whereas this difference was not present at 72 hours. We hypothesize that the transient nature of this effect is related to the proteolytic effect of Der p 1, a major allergen in the HDM extract.<sup>40</sup> It has been shown that Der p 1 can temporarily break down TJs, which recover after removal of Der p 1 and closing of the barrier.<sup>41</sup> When HDM-challenged mice were pretreated with FP, the FD4 passage was significantly lower 1 and 24 hours after the last HDM challenge compared with that seen in sham-treated HDM mice. It still needs to be explored whether the effect of FP on FD4 passage is orchestrated by the anti-inflammatory effect or the barrier-promoting effect. However, BAL eosinophil counts are significantly increased 24 and 72 hours, but not 1 hour, after the last challenge, suggesting that the barrier effect comes first and is at least not enhanced by the highly inflammatory environment at 72 hours (see Fig E3 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)).

In summary, an impaired epithelial barrier function is found in patients with HDM-induced AR and is associated with lower occludin and ZO-1 expression. IL-4 decreased epithelial barrier function, which was prevented by FP. INs restored epithelial barrier integrity to similar levels found in control subjects and might be related to upregulation of TJ proteins.

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### Key messages

- The epithelial barrier is impaired in patients with HDM-induced AR.
- FP prevented IL-4-induced barrier dysfunction.

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## METHODS

### Isolation of primary NECs

Tissue was washed in sterile saline and enzymatically digested in 0.1% Pronase (Protease XIV, Sigma) solution in DMEM-F12 culture medium supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and 2% Ultrosor G (Pall Life Sciences, Zaventem, Belgium). After overnight incubation at 4°C while shaking, the protease reaction was stopped by the addition of FCS (10%). Cells were washed in culture medium and pelleted by means of centrifugation for 5 minutes at 100g. Cells were then resuspended in 10 mL of culture medium and incubated in a plastic culture flask for 1 hour at 37°C to remove fibroblasts. The cell suspension was mixed with  $2 \times 10^7$  prewashed CD45 and CD15 magnetic beads (Dynabeads; Invitrogen, Merelbeke, Belgium), and epithelial cells were purified by means of negative selection, according to the manufacturer's instructions. Cell purity was verified by using cytospin preparations and was found to be 98% or greater.<sup>E1</sup>

### Ussing chamber experiments for evaluation of mucosal explant integrity

Nasal biopsy specimens were mounted in Ussing chambers with an opening of 0.017 cm<sup>2</sup> (Mussler Scientific Instruments, Aachen, Germany). Mucosal and serosal compartments were filled with 3 mL of 10 mmol/L glucose in Krebs-Ringer bicarbonate buffer. Solutions were kept at 37°C and continuously carbogenated with O<sub>2</sub>/CO<sub>2</sub> (95/5%). Experiments were performed in open-circuit conditions. Transtissue resistance was calculated from the voltage deflections induced by bipolar constant-current pulses of 16 mA every 6 seconds with a duration of 200 ms and was recorded for 2 hours. The average of all time points was taken, and results are presented as  $\Omega \times \text{cm}^2$ . FD4 (1 mg/mL, Sigma-Aldrich) was added to the mucosal compartment. Serosal samples were collected every 30 minutes over 2 hours to evaluate the fluorescence intensity with a fluorescence reader (FLUOstar Omega). Because a paracellular probe needs time to migrate to the serosal side, time points of 0 and 30 minutes were left out of the analysis. The average of the 60-, 90-, and 120-minute time points of the biopsy specimens were taken, and passage of FD4 is presented as picomoles.

### RT-qPCR for TJ genes

At day 21 in ALI culture, primary NECs were collected from the Transwell inserts with trypsin/EDTA and homogenized in lysis buffer from the Qiagen Mini RNeasy Kit (Qiagen, Germantown, Md). Nasal mucosal biopsy

specimens were snap-frozen immediately after isolation. First-strand cDNA was reverse transcribed with SuperScript II (Invitrogen, Carlsbad, Calif) with 1 µg of total RNA, according to the manufacturer's instructions. RT-qPCR was performed for claudin-1, claudin-4, occludin, and ZO-1 and the housekeeping genes encoding  $\beta$ -actin (*ACT2B*) and  $\beta_2$ -microglobulin. cDNA plasmid standards, consisting of purified plasmid DNA specific for each target, were used to quantify the target gene in the unknown samples, as previously described.<sup>E2</sup> Primer sequences can be found in Table E1.

### Immunofluorescence staining of TJs

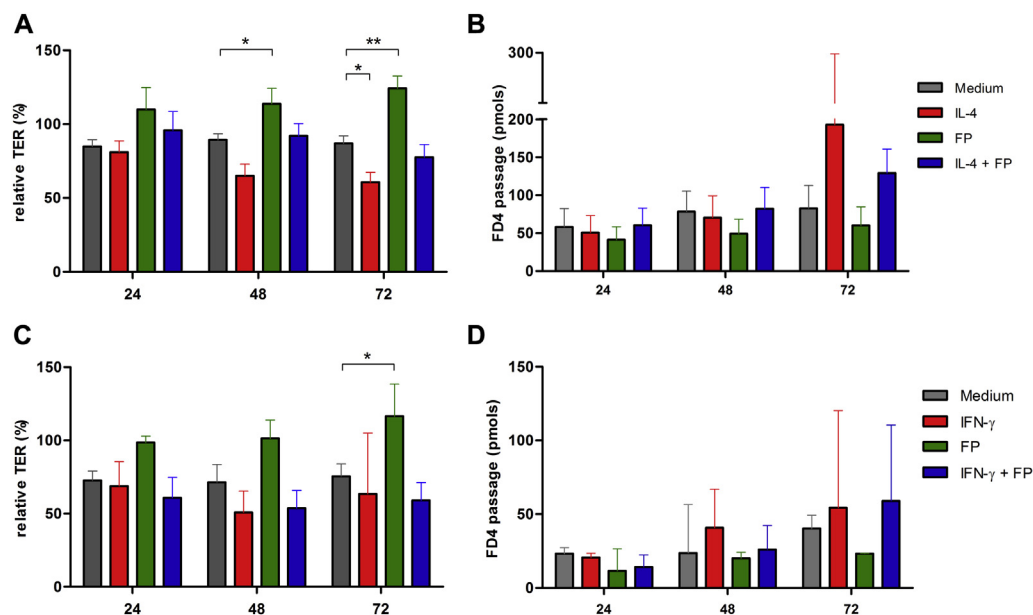
ALI cultures were cooled at 4°C and washed with PBS before overnight fixation with methanol at -20°C. Acetone was added for 1 minute, and ALI cultures were air-dried. ALI cultures were hydrated in IMF buffer (0.1% Triton X-100, 0.15 mol/L NaCl, 5 mmol/L EDTA, 20 mmol/L HEPES, and 0.02% NaN<sub>3</sub>) and incubated with mouse anti-ZO-1 (1:50, Invitrogen) and rabbit anti-occludin (1:25, Invitrogen). Donkey anti-mouse Alexa Fluor 488 and donkey anti-rabbit Alexa Fluor 594 (both 1:1000, Invitrogen) were used as secondary antibodies.

For immunofluorescence staining of nasal biopsy specimens, paraffin-embedded tissue slides (5 µm) were subjected to antigen retrieval in citrate buffer, pH 6 (Fluka). Antibodies used for immunofluorescence were as follows: anti-occludin (rabbit, polyclonal, 1:100, 31721; Abcam, Cambridge, United Kingdom), anti-ZO-1 (rabbit, polyclonal, 1/100, 61-7300, Invitrogen), and secondary antibody goat anti-rabbit Alexa Fluor 488 (1/2000, Invitrogen). After staining, tissues were mounted with 4'-6-diamidino-phenylindole dihydrochloride-containing mounting media. Stained slides were stored at -20°C in the dark.

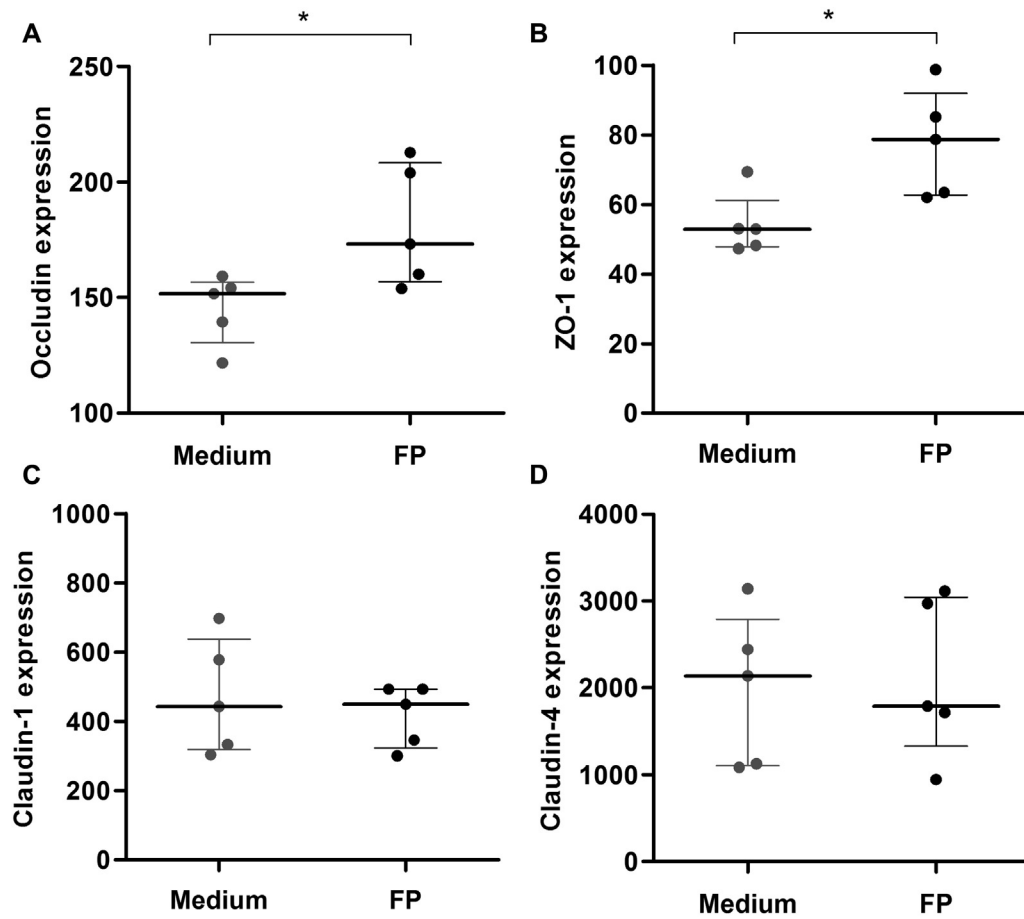
Confocal images were taken with either an LSM510 Meta Laser Scanning microscope (Zeiss, Oberkochen, Germany) for the *in vitro* data or a Leica TCS SPE confocal microscope (Leica Microsystems, Heerbrugg, Switzerland) for explants. IMARIS software (Bitplane, Zurich, Switzerland) was used for visualization of TJs.

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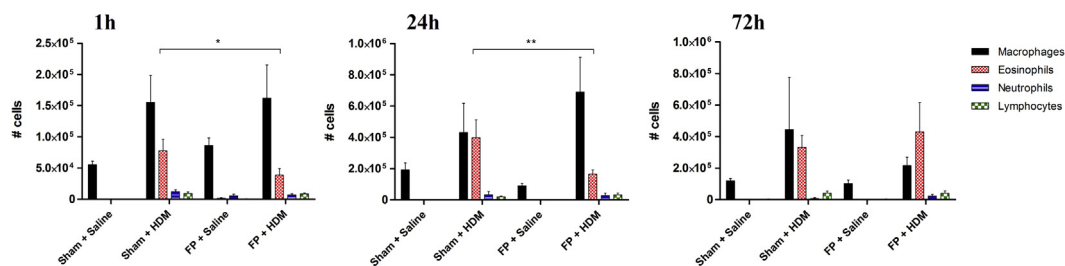
- E1. Bobic S, van Drunen CM, Callebaut I, Hox V, Jorissen M, Fokkens WJ, et al. Dexamethasone-induced apoptosis of freshly isolated human nasal epithelial cells concomitant with abrogation of IL-8 production. *Rhinology* 2010;48:401-7.
- E2. Seys SF, Grabowski M, Adriaensen W, Decraene A, Dilissen E, Vanoirbeek JA, et al. Sputum cytokine mapping reveals an 'IL-5, IL-17A, IL-25-high' pattern associated with poorly controlled asthma. *Clin Exp Allergy* 2013;43:1009-17.



**FIG E1.** Regulation of epithelial barrier function *in vitro* by IL-4, IFN- $\gamma$ , and FP in patients with HDM-induced AR. NECs were isolated from the inferior turbinates of patients with HDM-induced AR ( $n = 5$ ) and cultured *in vitro* to evaluate the effect of proinflammatory cytokines and FP on NEC integrity. **A**, Effect of IL-4, FP, and IL-4 plus FP on epithelial integrity, as measured with TER in duplicate. **B**, Effect of IL-4, FP, and IL-4 plus FP on FD4 epithelial permeability. **C-D**, IFN- $\gamma$  did not affect TER and FD4 measurements. \* $P < .05$  and \*\* $P < .01$ .



**FIG E2.** FP upregulates mRNA expression of occludin and ZO-1 in primary NEC cultures from healthy control subjects ( $n = 5$ ) when stimulated with FP for 72 hours. FP increased mRNA expression of occludin (**A**) and ZO-1 (**B**) and had no effect on claudin-1 (**C**) and claudin-4 (**D**) expression. Relative mRNA expression versus that of the housekeeping genes encoding  $\beta$ -actin and  $\beta_2$ -microglobulin. Data are presented as medians and interquartile ranges.  $*P < .05$ .



**FIG E3.** Differential cell counts in bronchoalveolar lavage fluid in HDM-challenged mice. Eosinophilic bronchoalveolar lavage counts were significantly lower 1 and 24 hours after the last challenge between sham/HDM-challenged and FP/HDM-challenged mice. After 72 hours, no difference was found in eosinophilic cell counts. \* $P < .05$  and \*\* $P < .01$ , Kruskal-Wallis test with *post hoc* analysis.



**TABLE E1.** Primer and probe sequences used for RT-qPCR

	Forward primer	Reverse primer	Probe
$\beta$ -Actin	gga cat ccg caa aga cct gt	ctc agg agg agc aat gat ctt gat	ctg gcg gca cca cca tgt acc ct
$\beta_2$ -Microglobulin	ctg aag ctg aca gca ttc gg	ctt tgg agt acg ctg gat agc c	aga tgt ctc gct ccg tgg cct tag c
Claudin-1	cca gtc aat gcc agg tac gaa t	ata ggg cct tgg tgt tgg gt	tca ggc tct ctt cac tgg ctg ggc
Claudin-4	ggt ctg ctc aca ctt gct gg	gac gga ctt aac gtt cgc aga g	tgg ctt tat ctc ctg act cac ggt gca
Occludin	cca atg tcg agg agt ggg tta a	ttg cca ttg gaa gag tat gcc	ctg cag gca cac agg acg tgc c
ZO-1	gtg cct aaa gct att cct gtg agt c	cta tgg aac tca gca cgc cc	tgg cca cag ccc gag gca tat t